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TITLE Improved Method for Measuring Membrane Potential

INVENTORS:

Dieter Kalubert
250 Blue Sky Drive
Arroyo Grande, CA 93420
A Citizen of Canada

Zhenjun Diwu
855 Cascade Drive
Sunnyvale, CA 94087
A Citizen of the People's Republic of China

Guoliang Yi
1007 Valley Forge Drive
Sunnyvale, CA 94087
A Citizen of the People's Republic of China

Martin Kirk
712 Dartmouth Avenue
San Carlos, CA 94070
A Citizen of the United States of America

ASSIGNEE:

Molecular Devices Corporation
1311 Orelans Drive
Sunnyvale, CA 94089-1136

IMPROVED METHOD FOR MEASURING MEMBRANE POTENTIAL

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to the fields of biology and chemistry, and bioanalytical instrumentation. In particular, the present invention is directed to composition and methods for use in sensing membrane potentials, especially in biological systems. Potentiometric optical probes enable researchers to perform membrane potential measurements in organelles and in cells that are too small to allow the use of microelectrodes. Moreover, in conjunction with imaging techniques, these probes can be employed to map variations in membrane potential across excitable cells and perfused organs with spatial resolution and sampling frequency that are difficult to achieve using microelectrodes.

BACKGROUND OF THE ART

The plasma membrane of a cell typically has a transmembrane potential of approximately –70 mV (negative inside) as a consequence of K^+, Na^+ and Cl^- concentration gradients that are maintained by active transport processes. Potentiometric probes offer an indirect method of detecting the translocation of these ions. Increases and decreases in membrane potential (referred to as membrane hyperpolarization and depolarization, respectively) play a central role in many physiological processes, including nerve-impulse propagation, muscle contraction, cell signaling and ion-channel gating (references 1-3). Potentiometric probes are important tools for studying these processes, and for cell-viability assessment. Potentiometric probes include the cationic or zwitterionic styryl dyes, the cationic carbocyanines and rhodamines, the anionic oxonols and hybrid oxonols and merocyanine 540 (references 4-8). The class of dye determines factors such as accumulation in cells, response mechanism and toxicity. Mechanism for optical sensing of membrane potential have traditionally been divided into two classes: sensitive but slow redistribution of permanent ions from extracellular medium into the cell, and fast but small perturbation of relatively impeable dyes attached to one face of the plasma membrane (references 2 and 3).

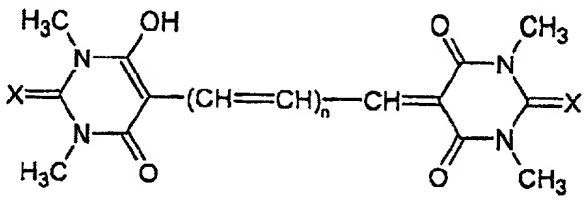
The bis-barbituric acid and thiobarturic oxonols, often referred to as DiBAC and DiSBAC dyes respectively, form a family of spectrally distinct potentiometric probes with excitation maxima covering most range of visible wavelengths. $DiBAC_4(3)$ and $DiSBAC_2(3)$ have been the two most popular oxonol dyes for membrane potential measurement (references 9 and 11). These dyes enter depolarized cells where they bind to intracellular proteins or membranes and exhibit enhanced fluorescence and red spectral shifts. Increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. $DiBAC_4(3)$ reportedly has the highest voltage sensitivity. The long-wavelength $DiSBAC_2(3)$ has frequently been used in combination with the UV light–excitable Ca^{2+} indicators Indo-1 or Fura-2 for simultaneous measurements of membrane potential and Ca^{2+} concentrations. Interactions between anionic oxonols and the cationic K^+-valinomycin complex complicate the use of this ionophore to calibrate potentiometric responses. DiBAC and DiSBAC dyes are excluded from mitochondria because of their overall negative charge, making them superior to carbocyanines for measuring plasma membrane potentials.

In general, DiBAC and DiSBAC dyes bearing longer alkyl chains had been proposed to have better propertie for measuring membrane potentials (references 5 and 12). $DiSBAC_6(3)$ has been selected to use in a FRET-based membrane potential assay (reference 12). There are no reports on $DiBAC_1$ and $DiSBAC_1$ for measuring membrane potentials.

It has been discovered that $DiBAC_1(3)$ and $DiSBAC_1(3)$ that possess unexpected properties that can be used to measure membrane potentials with FLIPR and other fluorescence devices. Compared with other members of DiBAC and DiSBAC family, $DiBAC_1(3)$ and $DiSBAC_1(3)$ give stronger signal and faster response besides its better water solubility.

SUMMARY OF THE INVENTION

The invention encompasses an improved method for measuring membrane potential using compounds of the formula I as potentiometric probes. These probes may be used in combination with other fluorescent indicators such as Indo-1, Fura-2, and Fluo-3, calcium green or Fluo-4. Such probes may be used in microplate reading devices such as FLIPR, fluorescent imaging plate reader,sold by Molecular Device Corp., of Sunnyvale, CA; flow cytometers; and fluorometers. Such probes are used to measure membrane potential in live cells.



Wherein X is O, or S; n is 1 or 2.

I

The invention also encompasses test kit containing reagents of compound I, compound I in combination with a fluorescent reagent and in particular fluorescent indicators such as Indo-1, Fura-2, Fluo-3, calcium green or Fluo-4.

Another aspect of the invention involves a method for generating voltage sensitive fluorescent changes comprising incubating the membrane with:

(a) A first reagent selected from the potentiometric probes which redistribute from one side of the membrane to the opposite side in response to transmembrane potential; and a second reagent selected from the group consisting of non-fluorescent dyes or pigments that re not

membrane-permeable, and undergo energy transfer with the first reagent one side of the membranes to reduce or eliminate the fluorescence signal on that side; or

(b) A first reagent selected from the potentiometric probes which redistribute from one side of the membrane to the opposite side in response to transmembrane potential; and a second reagent selected from the group consisting of non-fluorescent dyes or pigments that re not membrane-permeable, and absorb the excitation light or emission from the first reagent one side of the membranes to reduce or eliminate the undesired fluorescence signal; or

(c) A first reagent selected from the potentiometric probes which redistribute from one side of the membrane to the opposite side in response to transmembrane potential; and a second reagent selected from the group consisting of fluorescent or luminescent probes which undergo energy transfer with the first reagent, said second reagent being located adjacent to either the one side or the other side of the membrane.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a reaction scheme for making DiBAC and DISBAC.

Figure 2 illustrates the voltage-dependent fluorescence intensity changes of $DiSBAC_1$ (3) in P2X2 cells at different ATP concentrations.

Figure 3 illustrates the absorption spectra of $DisBAC_1(3)$, $DIsBAC_2(3)$, $DisBAC_4(e)$, $DiBAC_1(3)$ and $DiBAC_4(3)$ in 1:1 methanol/water.

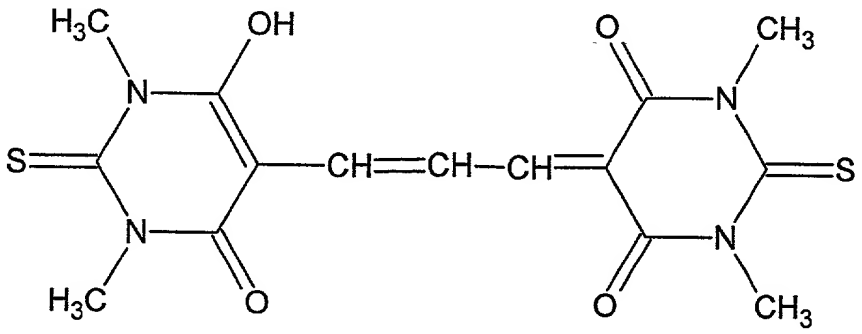
Figure 4 illustrates the fluorescence spectra of $DisBAC_1(3)$, $DisBAC_2(3)$, $DisBAC_3(3)$, $DiBAC_4(3)$ $DiBAC_1(3)$ and $DiBAC_4(3)$ in 1:1 methanol/water.

DETAILED DESCRIPTION OF THE INVENTION

Compounds useful in practicing the present invention are made according to methods described by G.W. Fischer in Chem Ber, 1969, 102:2609-2620, as shown in Figure 1.

The invention is illustrated by the following examples:

Example 1. Preparation of $DiSBAC_1(3)$



DiBAC and DiSBAC dyes are prepared based on the procedure of ethyl and butyl derivatives) (H. Bartsch and G. Haubold, Arch. Pharm. 1982, 315, 761-766). Specifically, malonaldehyde bis(phenylimine)monohydrochloride (2.6 g, 10 mmol) and 1,3-dimethyl-2-thiobarbituric acid (3.5 g, 20 mmol) are dissolved in acetonitrile (40 mL). To the solution is added triethylamine (2 g, 20 mmol). The reaction mixture is refluxed until the starting materials are completely consumed as indicated by TLC. The mixture is cooled to room temperature, and poured into acidic water (pH 2-3, 350 mL). The resulting suspension is filtered to collect the solid that is washed with cold water and air-dried. The crude product is further purified on a silica gel column using a gradient of dichoromethano/metahnol to give the desired product.

$DiSBAC_1(5)$, $DiBAC_1(3)$, $DiBAC_1(5)$ and other oxonol dyes are prepared analogous to the above procedure.

Example 2. Measuring membrane potentials using $DiSBAC_1(3)$ in combination with the fluorescence imaging plate reader (FLIPR™)

This specific example illustrates how to use $DiSBAC_1(3)$ in P2X2 cells in combination with FLIPR™, fluorescent imaging plate reader sold by Molecular Devices Corp. of Sunnyvale, CA. P2X2 cells are 1321 N1 astrocytoma cells transfected to overexpress the purinergic P2X2 ligand-gated ion channel. P2X2 belongs to a class of purinergic ion channels that pass calcium and sodium in response to purine, including adenosine 5'-triphosphate (ATP). P2X2 cells are propagated and maintained in DME (high glucose), 10% FCS, 1X Pen/Strep and 2mM L-glutamine. Doubling time is approximately 36 hours. P2X2 cells should be split at a 1 to 2 ratio upon confluence. The cells should be carried for no more than 20 passages. When approaching this limit, a new frozen vial of cells should be resurrected. Following is a typical kit procedure:

1. Plate 40,000 P2X2 cells in 100μL per well for 96 well plates or 10, 000 P2X2 cells in 25μL per well for 384 well plates for overnight.
2. Prepare 1X Loading Buffer.

2.1 To prepare the 1X Assay Buffer, pipette 10mL of 10X Reagent Buffer (1X Hanks' Balance Saline Solution + 20 mM HPEPES, pH 7.40) and dilute in 90 mL of distilled water. Adjust pH to 7.4 using 1.0 N NaOH and/or 1.0N HCl.

2.2 To prepare 10 mM stock solution of $DiSBAC_1(3)$, dissolve 3.8 mg $DiSBAC_1(3)$ in 1 mL DMSO

2.3 To prepare 10% pluronic acid, dissolve 400 mg pluronic acid in 4 mL water. Heat in 37°C to complete dissolution.

2.4 To prepare 1X Loading Buffer, add 30 μL of stock $DiSBAC_1(3)$, 8 μL 10% pluronic acid, and 20.0 mg DB71 in the 1X FLIPR assay buffer.

3. Load Cells with 1X Loading Buffer
4. Remove cell plates from the incubator
5. Add 100μL of 1X Loading Buffer per well of 96-well plates or 25 μL per well of 384-well plates.
6. Incubate plates at 37 °C for 30 minutes.
7. Run the FLIPR Membrane Potential Assay

7.1 Make 5X compound plate prior to running the FLIPR assay. Dissolve 27.5 mg of ATP (Sigma Cat# A3377) in 1 mL of sterile water to make a 50 mM stock solution. Make appropriate dilutions for 100 nM, 1 uM and 10 uM and transfer a minimum of 200 μL to each well of a compound plate.

7.2 Confirm that Membrane Potential Filter is installed. Choose p2x2.fcf for experimental setup in the FLIPR software. Set up the appropriate experiment parameters.

7.3 After incubation, transfer the plates directly to FLIPR and begin the Membrane Potential Assay.

8. Run FLIPR and perform data analysis.

Curve of the ATP dose response should look similar to that shown in Figure 2; an initial depolarization event depicted as an increase in fluorescence followed by repolarization or decay in signal near baseline. Also, EC50 should be approximately in the 10-100 nM range.

Example 3. Measuring membrane potentials using $DiBAC_1(3)$ in combination with a microscope

$DiBAC_1(3)$ is used to measure membrane potential change with a microscope as described by L. M. Loew (Methods in Cell Biology, vol. 38, pp195-209).

Example 4. Measuring membrane potentials using $DiSBAC_1(3)$ in combination with flow cytometer

$DiBAC_1(3)$ is used to measure membrane potential change with a flow cytometer as described by L. M. Loew (Methods in Cell Biology, Vol. 41, Part A, pp195-209).

Example 5. Water solubility and hydrophobicity comparison of DiBAC and DiSBAC dyes

$DisBAC_1(3)$, $DisBAC_2(3)$, $DisBAC_3(3)$, $DisBAC_4(3)$, $DiBAC_1(3)$ and $DiBAC_4(3)$ are dissolved in DMSO (3 mM). The DMSO stock solutions are respectively partrioned in 1:1 octanol/water mixture. The concentrations of the oxonol dyes in octanol and water layers are determined by absorption spectra. The results are summarized in the following table I. As shown in the table, $DisBAC_1(3)$ and $DisBAC_1(3)$ are much more hydrophilic than the other oxonol dyes. They also have much better water solubility.

Compound	λ_{max} in octanol (nm):	Absorbance in Water:	Absorbance in Octanol:	Absorbance in Water/ Absorbance in Octanol	Relative Values:
$DiBAC_1(3)$	495	1.297	0.3104	4.179	1
$DiBAC_4(3)$	497	0.002501	2.854	0.00088	0.00021
$DiSBAC_1(3)$	538	0.06147	0.9126	0.0674	1
$DiSBAC_2(3)$	543	0.01143	2.187	0.0052	0.078
$DiSBAC_3(3)$	544	0.002256	4.331	0.0005	0.0077
$DiSBAC_4(3)$	544	0.003318	3.226	<0.0010	<0.0077

Table 1

Example 6. Absorption comparison of DiBAC and DiSBAC dyes

$DisBAC_1(3)$, $DisBAC_2(3)$, $DisBAC_3(3)$, $DisBAC_4(3)$, $DiBAC_1(3)$ and $DiBAC_4(3)$ are dissolved in methanol (1 mM). The stock solutions are diluted with 1:1 methanol/water, and the absorption spectra are recorded in a spectrophotometer. As shown in Figure 3, $DiBAC_1(3)$ and $DisBAC_1(3)$ possess unexpected blue shift compared to the other oxonol dyes.

Example 7. Fluorescence comparison of DiBAC and DiSBAC dyes

$DisBAC_1(3)$, $DisBAC_2(3)$, $DisBAC_3(3)$, $DisBAC_4(3)$, $DiBAC_1(3)$ and $DiBAC_4(3)$ are dissolved in methanol (1 mM). The stock solutions are diluted with 1:1 methanol/water, and the absorption spectra are recorded in a fluorometer. As shown in Figure 4, $DiBAC_1(3)$ and $DisBAC_1(3)$ possess unexpected blue shift compared to the other oxonol dyes.

Example 8. Fluorescence response comparison of DiBAC and DiSBAC dyes for sensing membrane potentials in FLIPR assays

$DisBAC_1(3)$, $DisBAC_2(3)$, $DisBAC_3(3)$, $DisBAC_4(3)$, $DiBAC_1(3)$ and $DiBAC_4(3)$ are dissolved in DMSO (1 mM). The stock solutions are respectively used to assay membrane potential changes in the P2X2 cells in combination with FLIPR™ as described in Example 2. The results are summarized in the following table 2.

Compounds	Fluorescence enhancement by 10 μM ATP stimulation (in folds)	Response speed
$DiBAC_1(3)$	3.7	fast
$DiBAC_4(3)$	3.3	slow
$DisBAC_1(3)$	108.5	fast
$DisBAC_2(3)$	38.6	moderate
$DisBAC_3(3)$	14.5	slow
$DisBAC_4(3)$	2.3	slow

Table 2

As shown in the table 2, $DisBAC_1(3)$ is much more sensitive, and has faster response to membrane potential change than the rest DisBACs. $DiBAC_1(3)$ also has much faster response to membrane potential change than $DiBAC_4(3)$.

Example 9. Use of $DiSBAC_1(3)$ as a Fluorescent Indicator of Transmembrane Potential

Depolarization of PC 12 Cells

Protocols for transmembrane potential measurements are summarized briefly since they are similar to those given in detail in Example I above. The Bis-(1, 3-dimethylthiobarbituric acid) trimethine oxonol, [$DiSBAC_1(3)$], fluorescent reagent may be purchased from Molecular Probes (Eugene, OR, USA). The 1X Cell-Loading Buffer for $DiSBAC_2(3)$, consists of sodium-free Tyrode's Buffer (SFTB), 2.5uM $DiSBAC_1(3)$, and 200 uM Direct Blue 71 (as the fluorescence quencher).

A rat pheochromocytoma (adrenal) cloned cell line, PC12, is grown in RPMI 1640 culture medium with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (PS), 2 mM L-

glutamine, and 1mM sodium pyruvate. Cells were grown in suspension, and subsequently centrifuged from growth medium and resuspended in $DiSBAC_2(3)$], 1X Cell-Loading Buffer. Approximately 100,000 cells were plated per well in a 96 well microtiter plate pre-coated with poly-D-lysine to enchance cell adhesion, centrifuged at 1000 rpm for 4 minutes, and placed in an incubator for an additional 20 minutes. Cells were not washed with any liquid medium, nor was the 1X Cell-Loading Buffer removed prior to performing fluorescence measurements.

The fluorescently labeled cells were analyzed for changes in membrane potential by using FLIPR™ fluorescent imaging plate reader. Briefly, cells were depolarized with addition of 75 mM potassium gluconate in sodium containing Tyrode's Buffer (SCTB). To inhibit voltage-gated sodium channels cells were previously incubated with 100 uM tetrodotoxin (TTX) for 5 minutes prior to depolarization. The data reveal that cell-depolarization (due to potassium addition) causes increased $DiSBAC_1(3)$ fluorescence. Inhibition of sodium channels by TTX results in smaller changes in membrane potential upon potassium addition as indicated by a smaller increase in fluorescence as compared to the positive control (75 mM potassium gluconate without TTX).

The above examples illustrate the present invention and are not intended to limit the invention in spirit of scope.

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